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ORIGINAL ARTICLE

Virulence Determinants among Extended-Spectrum B- Lactamases producers of Uropathogenic Escherichia coli isolates In Zagagig University Hospitals, Egypt

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Uropathogenic E coli, Virulence determinants, ESBL, PCR

ABSTRACT

Background: *Uropathogenic* E. coli *(UPEC), the major causative agent of urinary tract infections (UTI) worldwide have variant virulent mechanisms. UPEC resistance to commonly used antibiotics represents a major health problem all over the world. The empiric antibacterial therapy of the infections should be based on a local experience of the susceptibility and the resistance profile.* ***Our objectives*** *are to detect the presence of and possible relation between virulence genes and ESBL production in E. coli strains isolated from patients with UTI in Zagazig, Egypt.* ***Methodology:*** *75 E. coli strains isolated from patients with UTI were screened for virulence genes: fimH (type 1 fimbriae), pap (pyelonephritis-associated pili), sfa/foc (S and F1C fimbriae), afa (afimbrial adhesins), hly (hemolysin), cnf1 (cytotoxic necrotizing factor), aer (aerobactin), traT (transfer gene foe serum resistance) by polymerase chain reaction (PCR) and for antimicrobial resistance by disc diffusion method according to CLSI criteria along with extended spectrum B lactamases (ESBL) detection by double disc synergy (DDS) method.* ***Results:*** *The fimH gene was the most common virulence gene and was detected in 85.3% of the UTI isolates. The trat gene was present in 69.3% (52/75) and the pap gene in 52% of isolates. The aer gene was detected in 40% while the sfa gene and afa gene were shown in 29.3% and 21.3% of isolates respectively. Among the genes coding for toxin, hly was found in 9.3%, while cnf was present in only 8% of isolates. Eight strains genes lacked any tested virulence markers accounting (10.7%). Out of 75 strains, 41 (54.7%) were ESBL producers. Statistically significant relationships were found between fimH, tratT and pap with ESBL production (p values of <0.05, <0.0001 & <0.0001 respectively).* ***Conclusions****: There was a significant association between some virulence determinants of UPEC and ESBL production, these determinants may contribute to a better medical intervention. Also we need the used treatment regimens to be revised to reach better therapeutic effects*.

INTRODUCTION

Urinary tract infection UTI is the most frequent human bacterial infection encountered at all ages all around the world, about 50% of women and 12% of men get UTI during their life, recurrent in 20% to 30% of women. Consequently, it is a major public health matter[1]. *Escherichia coli* strains causing disease outside the gastrointestinal tract belong to a various group of isolates known to be uropathogenic *E. coli* (UPEC) which are responsible for 50-80% of UTI [2].

UPEC strains carry different virulence factors that contribute to the colonization and the survival in the normal urinary tract resulting in development of the infectious process.The generally accepted hypothesis is that UPEC evolved from non-pathogenic strains by acquiring new virulence factors from auxiliary DNA horizontal transfer [3].

UPEC strains harbor numerous types of virulence factors such as adhesins, toxins and iron uptake systems that facilitate colonization and persistence of the bacteria in the urinary tract [4].Attachment of the bacterium to the uroepethelium is the essential stage to initiate and develop UTI. This process allows bacteria to resist the flushing action of the urine flow and bladder emptying, thus, promoting bacterial persistence and activation of the host signaling pathways [5]. Various types of adhesins are produced including type 1 fimbriae, coded by the *fim* gene cluster; P fimbriae, coded by *pap* (pyelonephritis-associated pili) genes; S fimbriae, coded by *sfa* genes; and Afa adhesins, coded by *afa* genes, for afimbrial adhesins. Some studies revealed that *afa, pap*

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and *sfa* could perform as predictors of cystitis and/or pyelonephritis[5].

Other important virulence distinctive of UPEC is serum resistance; the ability that protects bacteria against bactericidal activity of serum so bacteria can persist in body fluids. This factor is encoded by *traT* gene [6].

Production of toxins such as hemolysin and cytotoxic necrotizing factor 1 (CNF1) causes tissue damage enabling bacterial dissemination, releasing of host nutrients, and may also modify host signaling pathways affecting several processes, such as inflammatory responses, host cell survival, and cytoskeletal dynamics[7].

Another feature of UPEC is the expression of iron acquisition systems that utilize siderophores to scavenge iron from the environment and afterwards concentrate it in the bacterial cytosol, it is essential for the ability to colonize and persist in host iron-poor niches such as the urinary tract since limiting iron availability is an important host defense against invading bacterial pathogens [7].

The principal objective of early recognition and treatment of UTI is the prevention of renal parenchymal damage and subsequent renal scarring, so, identification of virulence factors can be useful for diagnosis and therapeutic strategies. Studies show that antibiotic resistance is increasing among UPEC strains every year. The high antimicrobial resistance of UPEC significantly reduces the therapeutic options and increases the treatment costs and mortality rates [8].

The aim of this study is to evaluate the occurrence of different genes coding for virulence factors among UPEC isolated from urine of patients with UTI from Zagazig University Hospitals and to study the association of these genes with ESBL production.

METHODOLOGY

1. Study Design and Patient Selection

A cross-sectional study was carried out at Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, Egypt from June 2014 to June 2015. Patients already admitted to Zagazig University Hospitals complaining of symptomatic UTI were enrolled in the present study. The patients showed one of the following symptoms: fever (>38°C), dysuria, frequency, urgency. All enrolled patients had significant bacteriuria ($>10^5$ cfu/ml). Informed consent was obtained from each patient before the commencement of the study. Exclusion criteria were: a) Patients with any anatomical or functional deficiencies in urinary tract function, b) Patient with indwelling catheters. This study was approved by the local institutional review board (Zagazig University IRB).

2. Samples processing and identification of *E.Coli* strains:

Clean catch mid stream urine samples were collected from UTI patients in sterile screw capped containers. Quantitative urine cultures were performed [9]. Urine samples were cultured on MacConkey agar (Oxoid Ltd., Basingstoke UK), lactose fermenting colonies were identified by biochemical reaction tests for identification of *E.coli*. [9]

3. Antimicrobial susceptibility testing

All isolates were subjected to antibiotic sensitivity testing by the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [10]. Sixteen antibiotics have been used in this study, supplied by (Oxoid Ltd., Basingstoke UK) including; ampicillin (10 µg), amoxicillin/clavulanic acid (20/10µg), piperacillin/tazobactam (100/10 µg), amikacin (10µg), gentamicin (10µg), cefeperazone (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefepime (30 µg), Azteronam (30 µg), ciprofloxacin (5 µg), levofloxacin (5µg), cotrimoxazole (25 µg), nitrofurantoin (300 µg) and Imipenem (10mg). The inhibition zone size was interpreted by using the standard recommendation of the Clinical Laboratory Standard Institute CLSI, 2012 [10]. In all reactions, the *E. coli* ATCC 25922 was used as quality control organisms

4. The ESBL Confirmatory Test

If any strain had zones of (Ceftazidime ≤ 22 mm or Aztreonam ≤27 mm or Ceftriaxone ≤25 mm or Cefotaxime ≤27 mm), this strain was indicated as potential ESBL producer. So, phenotypic confirmatory test; Double disc approximation or double disc synergy (DDS), was done and interpreted as described by CLSI[10]. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as nonESBL and ESBL producing organisms, respectively

5. Extraction of genomic DNA and detection of virulence markers for isolated *E.coli*:

DNA extraction was performed using an optimized heat shock method[3]. A template DNA stock was stored at 20ºC.

Specific primers were used to amplify sequences of the *fimH, pap, sfa, afa, hly, cnf, aer* and *traT* operons. Details of primer sequences and predicted sizes of the amplified products are shown in table 1. Detection of *pap, sfa, afa, hly, aer* and *cnf1* sequences was done by multiplex PCR. The PCR reaction mixture contained: 0.5 µL DNA (50 ng) in 24.5 µL Multiplex PCR Master Mix; QIAGEN®, Germany (containing: HotStarTaq® DNA Polymerase, Multiplex PCR Buffer, dNTP Mix, Q-Solution, 5x, RNAse-Free Water) and 5 µl 10x primer mix; 2 µM each primer (Midland Certified Reagent Company Inc, USA). The amplification was carried out in Biometra T gradient thermal cycler (Germany). Conditions consisted of 1 cycle of 94°C for 60 s; 30 cycles of 94°C for 60 s, 63°C for 30 s, 72°C for

90 s followed by a final cycle of 72°C for 300 s. Universal 16S rRNA gene was amplified with 1500 bp product in each PCR run as positive control [11]. PCR master mix without addition of primers or DNA was included in each PCR run as negative control. A 10 µl aliquot of the PCR product underwent gel electrophoresis on 2% agarose, stained with ethidium bromide solution. Amplified DNA fragments of specific sizes were detected by UV-induced fluorescence, and the size of the amplicons was estimated by comparing them with DNA MW-marker (iNtRON Biotechnology, Korea) included on the same gel.

Statistical analysis

The data of the study were analyzed by using the SPSS 17.0. The Chi-square test was used. $P<0.05$ was considered statistically significant.

Table 1: Primers used for detection of UPEC virulence genes

Target gene	*Primer Sequence (5'-3')*	*Product size*	*Reference*
fimH	AACAgCgATgATTTCCAgTTTgTgTg ATTgCgTACCAgCATTAgCAATgTCC	465	[12]
pap	GCAACAGCAACGCTGGTTGCATCAT AGAGAGAGCCACTCTTATACGGACA	336 bp	[13]
Sfa	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAACCTGGCA	410 bp	[13]
afa	GCTGGGCAGCAAACTGATAACTCTC CATCAAGCTGTTTGTTCGTCCGCCCG	750 bp	[13]
hly	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	1177 bp	[13]
aer	TACCGGATTGTCATATGCAGACCGT AATATCTTCCTCCAGTCCGGAGAAG	602 bp	[13]
Cnf1	AAGATGGAGTTTCCTATGCAGGAG CATTCAGAGTCCTGCCCTCATTATT	498 bp	[13]
traT	GGTGTGGTGCGATGAGCACAG CACGGTTCAGCCATCCCTGAG	290 bp	[14]

RESULTS

A total of 300 urine samples was considered for the urine culture. Significant bacteriuria was observed in 128 (42.7%) isolates. Moreover amongst the 128 isolates, *E. coli* was detected in 75 (58.6 %) isolates. In this study we observed a higher proportion of UTI in females (69.3%) than in males (30.7%). This is a consistent trend worldwide. The incidence of UTI in female patients with age group of 51–60 years (55.76%) was highest while the age group 21-30 years had the lowest incidence of UTI (9.6%). Among male patients, the highest prevalence of UTI was observed in the age group of 51–60 years (52.2%) and the lowest was seen in the age group of 31–40 years (13%).

The antimicrobial susceptibility patterns of these *E. coli* isolates against a spectrum of 16 selected antimicrobial agents of different classes were analyzed as shown in table 2. It was observed that all isolates were sensitive to imipenem. Susceptibility to cephalosporins was between 28 and 45%, which is quite low. Susceptibility to piperacillin/tazobactam, amoxicillin/clavulanic acid, amikacin, gentamycin, levofloxacin and ciprofloxacin were good representing 90.7%, 80%, 80%, 74.7%, 65.3% and 54.7% respectively.

Out of the 75 UPEC isolates, 54 isolates, that showed resistant to either all of ceftazidime, aztreonam, cefotaxime, and ceftriaxone or any one, were subjected to the ESBL confirmation test (DDS). Out of these 54 isolates, 41 isolates were ESBL producers. Antimicrobial sensitivity pattern of ESBL producing *E. coli* showed that it was 100% susceptible to imipenem, therefore, imipenem was excluded from statistical analysis. Comparison between antibiotic resistance pattern of the isolated ESBL UPEC and non ESBL strains revealed a significant statistical value to ciprofloxacin, aztreonam, ampicillin, Amoxicillin/ clavulanic acid, ceftriaxone and cefepime while there is insignificant statistical value (P-value > 0.05) between UPEC and non ESBL *E.coli* strains to other tested antibiotics (Table 3).

Table 2: Antibiotic susceptibility results for the 75 uropathogenic *E coli*

Antibiotics	***Resistant***		***Sensitive***	
	No.	**(%)**	**No.**	**(%)**
Ampicillin	50	66.7	25	33.3
Amoxicillin/clavulanic acid	15	20	60	80
Piperacillin/tazobactam	7	9.3	68	90.7
Amikacin	15	20	60	80
Gentamicin	19	25.3	56	74.7
Cefeperazone	52	69.3	23	30.7
Ceftriaxone	50	66.7	25	33.3
Ceftazidime	50	66.7	25	33.3
Cefotaxime	54	72	21	28
Cefepime	48	64	34	45.3
Azteronam	47	62.7	28	37.3
Ciprofloxacin	34	45.3	41	54.7
Levofloxacin	26	34.7	49	65.3
Cotrimoxazole	33	44	42	56
Nitrofurantoin	25	33.3	50	66.7
IImipenem	0	0	75	100

Table 3: Antibiotic resistance results for ESBL and non-ESBL producing of uropathogenic *E. coli*

Antibiotics	***ESBL producers (n=41)***		***Non ESBL producers (n=34)***		***P value***
	No.	**(%)**	**No.**	**(%)**	
Ampicillin	38	92.7	12	35.3	<0.0001
Amoxicillin/clavulanic acid	15	31.7	2	5.9	0.0057
Piperacillin/tazobactam	7	17	0	0	0.0122
Amikacin	11	26.8	4	11.8	0.108
Gentamicin	13	31.7	6	17.6	0.164
Cefeperazone	33	80	19	55.9	0.025
Ceftriaxone	36	87.8	14	41.2	<0.0001
Ceftazidime	32	78	18	52.9	0.0226
Cefotaxime	34	82.9	20	58.8	0.0216
Cefepime	34	82.9	14	41.2	0.0002
Azteronam	34	82.9	13	38	<0.0001
Ciprofloxacin	29	70.7	5	14.7	<0.0001
Levofloxacin	17	41.5	9	26.5	0.1772
Cotrimoxazole	18	43.9	15	44	0.993
Nitrofurantoin	17	41.5	8	23.5	0.102

Regarding the virulence genes, different frequencies were revealed among our UPEC as shown in table 4. The *fimH* gene was the most common virulence gene and it was detected in 85.3% (64/75) of the UTI isolates. The *traT* gene was present in 69.3% (52/75) of isolates and the *pap* gene in 52% of isolates. The *aer* gene was detected in 40% while the *sfa* gene and *afa* gene were shown in 29.3% and 21.3% of isolates respectively. Among the genes coding for toxin, *hly* was found in 9.3%, while *cnf1* was present in only 8% of isolates. Eight strains genes lacked any tested virulence markers and accounting 10.7%.

By comparing the prevalence of virulent genes between ESBL- producing and non-ESBL-producing UPEC, it was found that there was highly significantly difference regarding pap and *traT* ($p<0.0001$), the prevalence of *fimH* in ESBL producing UPEC was significantly higher than that in non ESBL producing UPEC (92.7% vs 76.5%, $p= 0.0497$). The prevalence of other virulent genes (except for *sfa* & *cnf1*) in ESBL producing UPEC seemed a higher results than non ESBL producing UPEC, however there was no statistically significant difference ($p>0.05$)> The results are shown in table 4 and figure 1.

DISCUSSION

E. coli is the cause of more than 80% of urinary tract infections in all age groups with serious complication if untreated for a long time or improperly

managed. Various virulence factors can be attributed to UPEC pathogenicity[3]. Better knowledge of virulence factors and its antibiotic resistance pattern helps clinicians to anticipate the development of infection hence better management will be possible. Surface virulence factors of UPEC are responsible for colonization of bacteria in the urinary tract [8].

In this study, out of 300 cultured urine samples 128 (42.7%) showed significant bacterial growth (>10^5 CFU-ml). Out of 128 isolates, 75 (58.6%) were identified as *E.coli* isolates. These results agree with another study in Egypt that detected *E coli* in 62.5% of their isolates[15]. Ranjan et al.[16] reported 68.6% of UPEC.

In our study, the antimicrobial susceptibility patterns of *E. coli* isolates against a spectrum of 16 selected antimicrobial agents of different classes were analyzed (table 2). All isolates were sensitive to imipenem, Susceptibility to cephalosporins was between 28 and 45%, which is quite low.

Table 4: Virulence genes among ESBL producers and non ESBL producers UPEC.

Virulence genes	*ESBL (n=41) n.(%)*	*Non ESBL(n=34) n.(%)*	*Total(n=75) n.(%*	*P value*
fimH	38(92.7)	26(76.5)	64(85.3)	0.0497
traT	40(97.6)	12(35.3)	52(69.3)	<0.0001
Pap	31(75.6)	8(23.5)	39(52)	<0.0001
Aer	19(46.3)	11(32.4)	30(40)	0.224
Sfa	10(24.4)	12(35.3)	22(29.3)	0.3053
Afa	10(24.4)	6(17.6)	16(21.3)	0.477
Hly	4(9.8)	3(8.8)	7(9.3)	0.883
Cnf1	3(7.3)	3(8.8)	6(8)	0.813

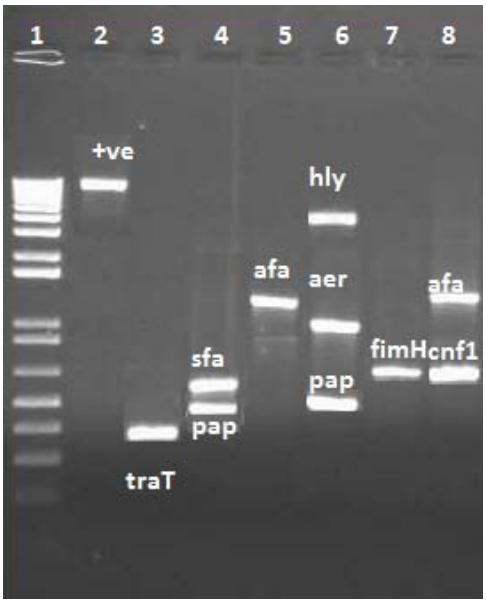

Fig. 1: Electrophoresis of PCR Product on Agarose Gel; Lane 1: 1500 bp molecular weight marker, lane 2: positive control, lanes 3-8 with different virulence genes detected in uropathogenic *E coli* isolates

Susceptibility to piperacillin/tazobactam, amoxicillin/clavulanic acid, amikacin, gentamycin, levofloxacin and ciprofloxacin were good representing 90.7%, 80%, 80%, 74.7%, 65.3% and 54.7% respectively.

The results in the present study come in agreement with other results with slight variation. In a study reported in Turkey by Nazik et al.[17], the UPEC isolates exhibited high resistance to amoxicillin-clavulanic acid, 77%, to trimethoprim/sulfamethoxazole 76%, ciprofloxacin 68%, gentamicin 51% and all isolates were found susceptible to imipenem. In a study done in Iraq, Merza and Jubrael [18] reported that imipenem was found as the most potent at all other antimicrobial agents with the resistance rate (4.7%), whereas ampicillin, and amoxicillin/clavulanic acid had the least effect on UPEC strains with resistant rates of 92.6%, and 90% respectively. The resistance patterns of ciprofloxacin, amikacin, gentamicin, and trimethoprim/ sulfamethoxazole were 52.6%, 46%, 70.7%, and 73.3% respectively, the resistant rates of cephalosporins ranging 71% to78%.

Also Tabasi et al. [19] found 100% of the isolated UPEC susceptible to imipenem in the study conducted in Iran, Where 7.61% of *E. coli* strains isolated by Habibian et al[20] were resistant to imipenem and it is so substantial, compared to high efficacy of imipenem for treatment of UTIs has been reported previously from same country[21]. Higher level of resistance in *E. coli* strains against imipenem has been reported by Poirel et al.[22]. This reported resistance to imipenem alarming clinicians in our country to adhere more stringently to the laboratory suggested antibiotic treatment for UTI to prevent the emergence of resistance to the last resort of antibiotics.

For many years, amoxicillin/clavulanate, cephalexin, trimethoprim/sulfamethoxazole or fluoroquinolones (for example, ciprofloxacin) were used as first line treatment of uncomplicated UTI. However, the wide spread resistance of *E. coli* to those antibiotics in many parts of the world excluded to be used for empiric therapy[23]. In almost all UTI cases, empirical antimicrobial treatment is initiated before the laboratory results of the urine culture are available. Misuse and self-medication in many countries including ours may be considered a major problem as antibiotics could be purchased without any prescription. Up to 95% of UTI cases are treated without bacteriological investigations[23].

In our study, using DDS as the ESBL phenotype confirmation test, 41 (54.7%) UPEC isolates were ESBL producers. In general, prevalence of ESBLs in *E. coli* in UTIs cases varies from country to country and from health institution to another, 75% by Padmavathy et al[24], and 52.4% by Yusuf et al. [25] were obtained on prevalence of ESBL enzymes detected in *E. coli* from UTI patients.

Antimicrobial resistance pattern of ESBL producing *E. coli* showed that it was 100% susceptible to imipenem, resistance rates were higher in ESBL producing UPEC than non-ESBL producing UPEC regarding all antibiotics (except for cotrimethoxazole), however, resistance to ciprofloxacin, aztreonam, ampicillin, Amoxicillin/clavulanic acid, ceftriaxone and cefepime showed significant statistical value (P-value <0.05) (Table 3). Al-Zarouni et al[26] reported high resistance rates to fluoroquinolones and cephalosporins and higher susceptibility rates to carbapenems and amikacin. Moreover, Maina et al[27] found a higher proportion of their isolates resistant to ciprofloxacin, levofloxacin, and approximately 100% sensitivity to carbapenems.

The widespread use of cephalosporins is the driving force behind the emergence of ESBL-producing organisms. It has been found that the genes that encode ESBLs are frequently found on the same plasmids as genes that encode resistance to aminoglycosides and trimethoprim- sulfamethoxazole conferring a particular challenge for the treatment of infections. The introduction and clonal expansion of competitive, resistant *E. coli* strains in the community is an important mechanism facilitating the increase in antimicrobial-resistant UTIs [28]

Regarding virulence genes, our results showed that genes coding for fimbrial adhesive systems represent the most common factors for the virulence of *E. coli* in UTI. The distribution of these genes in our strains were *fimH* (85.3%), *pap* (52%), followed by *sfa* (29.3%). A fimbrial adhesions *afa* was found in 21.3%. These results are in in agreement with other published data [4]. These results highlighted the crucial role of the virulence genes in *E. coli* causing UTI. This is in coincidence with Soto et al [29] who stated that the most important pathogenic factor of UPEC is the adhesion of to the uroepithelium that protects the bacteria from urinary discharge and promotes their ability to multiply and invade renal tissue.

However, the frequency of *pap* gene in UPEC isolates in the present study was different to other researchers[30,31]. The diversity in frequency of *pap* gene among different studies can be attributed to the fact various types of adhesins can be used by UPEC strains to bind to the urinary epithelial cells.

Usually, the invasive pathogens are highly resistant to the lethal activity of serum and the role of traT-protein in resistance of bacteria to serum is very important[8]. The current study showed that 69.3% of UPEC isolates contained *traT* gene. Oliveira et al.[4] showed that 76%of the UPEC were carrying *traT* gene and Kudinha et al. [32] and Neamati et al [8] reported a frequency of 77% and 74% respectively. These results are in accordance with those of the current study, which suggest that the *traT*, as a common and important virulence factor, could be considered as a target for therapeutic interventions.

Among our UPEC isolates, the prevalence of aerobactin operon *aer*, which confers the ability to acquire iron and the prevalence of *hly* and *cnf1* operons encoding two toxins implicated in tissue damage and dysfunction of local immune responses were 40%, 9.3% and 8% respectively. Oliveira et al. [4] found *aer* in (41%), *hly* in (5%) and *cnf1* in (18%). Different results were obtained by other researchers. [3,18]

These findings reflect the heterogeneity in the distribution of virulence genes among UPEC strains and the complex and multifactorial characteristic of UPEC virulence [4]

Our results showed that 8 UPEC lacked any tested virulence markers accounting (10.7%), Oliveira et al. [4] also found that 10% of UPEC strains lacked these virulence related genes. This result was explained by a scarce phenomenon suggesting the possibility of mutation at the level of the corresponding gene, leading to the absence of its detection. Therefore, a positive PCR shows the presence of the virulence gene, but a negative PCR does not point to the absence of the corresponding operon[3].

CONCLUSION

Since most of the UTIs are treated empirically, the selection of the antimicrobial agent should be determined, depending on both the most likely pathogen and its expected susceptibility pattern. Accordingly, it is essential to determine the local antimicrobial susceptibility patterns of the common uropathogens.

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